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Effects of a Capsule Containing *Clostridium butyricum*, 3-(4-Hydroxy-3-Methoxyphenyl)propionic Acid, and Salmon Milt-Derived DNA on Cognitive Function in Middle-Aged and Older Adults: A 12-Week Randomized, Double-Blind, Placebo-Controlled Trial

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Effects of a Capsule Containing *Clostridium butyricum*, 3-(4-Hydroxy-3-methoxyphenyl)propionic Acid, and Salmon Milt-Derived DNA on Cognitive Function in Middle-Aged and Older Adults: A 12-Week Randomized, Double-Blind, Placebo-Controlled Trial

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Abstract

Background: As the prevention of dementia onset and the slowing of its progression become a global challenge, nutritional interventions focusing on the gut-brain axis are garnering attention. This study examined the effects of a complex functional food containing *Clostridium butyricum*, 3-(4-hydroxy-3-methoxyphenyl)propionic acid (HMPA) derived from fermented rice bran, and salmon milt-derived DNA on cognitive function and bowel habits in middle-aged and older adults. **Methods:** A randomized, double-blind, placebo-controlled, parallel-group study was conducted on 80 men and women aged 55–79 years with subjective memory complaints and an MMSE-J score of 24 or higher (UMIN ID: UMIN000057405). Participants ingested either the Test food (containing 1.4×10^7 CFU of *C. butyricum*, 11.5 mg of HMPA, and 45 mg of salmon milt-derived DNA) or a Placebo once daily for 12 weeks. The primary outcome was cognitive function assessed by Cognitrax. Secondary outcomes included subjective cognition, mood state, bowel habits, blood biochemical parameters, and safety. A subgroup analysis was performed for participants aged ≥65 years with MMSE-J scores of 24–27. **Results:** In the overall analysis, no significant difference was observed in Composite Memory between groups; however, the number of correct responses in the Symbol Digit Coding (SDC) test, which is related to attention and processing speed, significantly improved in the Test food group. In the subgroup analysis, significant improvements in verbal memory-related indices were observed in the Test food group. Bowel habit indices showed no consistent between-group differences. Exploratory evaluations showed significant improvements in metabolic markers such as fasting plasma glucose, HbA1c, γ-GT, and uric acid in the Test food group. No serious adverse events occurred, and adherence to intake was high. **Conclusions:** Twelve-week intake of this complex functional food improved indices of attention and processing speed in the overall population, improved verbal memory indices in the high-risk subgroup, and positively affected metabolic markers. However, no clear effects on bowel habits were confirmed. Larger-scale and longer-term verification is needed in the future.

Keywords: *Clostridium butyricum*; HMPA; salmon milt-derived DNA; cognitive function; gut-brain axis; randomized controlled trial

1. Introduction

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The prevalence of dementia is increasing globally. As of 2024, it is estimated that over 55 million people worldwide suffer from dementia, a number predicted to rise further in the coming decades [1]. Dementia not only significantly impairs the individual's quality of life (QOL) but also imposes an immense social burden regarding care and medical costs; thus, preventing its onset and suppressing its progression are urgent international issues. In Japan, where the population of elderly individuals aged 65 and over is expected to increase, early-stage intervention is required to reduce social and medical burdens [2]. Indeed, estimates suggest that delaying the onset of dementia by just one year could reduce the global number of dementia patients by more than 6 million by 2050 [3], indicating that the social and economic significance of preventive intervention is extremely high.

In recent years, the bidirectional interaction between the gut microbiota and the central nervous system has been reported [4], attracting significant attention in neuroscience and nutrition as the "gut-brain axis." Gut bacteria produce short-chain fatty acids (such as butyrate) and neurotransmitter-like substances (such as GABA and serotonin precursors). These metabolites are thought to affect brain function via the vagus nerve, immune system, endocrine system, or the blood-brain barrier [5]. Furthermore, dysbiosis of the gut environment has been suggested to be associated with cognitive decline and the onset of psychiatric symptoms [5,6], with reports specifically linking it to neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [7,8]. Additionally, a decrease in butyrate-producing bacteria has been reported as one of the changes in the gut microbiota in Alzheimer's disease and mild cognitive impairment (MCI) [9,10]. Since butyrate, a short-chain fatty acid, can be involved in gut barrier function and immune/inflammation regulation [5], a decline in butyrate-producing capacity may be associated with the risk of cognitive decline via the gut-brain axis. Against this background, nutritional intervention targeting the gut microbiota is considered a novel strategy contributing to the maintenance of cognitive function [11].

In this study, we used a complex functional food containing *Clostridium butyricum*, 3-(4-hydroxy-3-methoxyphenyl)propionic acid (HMPA) derived from fermented rice bran, and salmon milt-derived DNA to examine the effects of its intake on cognitive function and bowel habits in middle-aged and older adults. Intervention with *C. butyricum* has been reported to improve cognitive decline via regulation of the microbiota-gut-brain axis in obesity models [12] and to improve cognitive function indices in Alzheimer's disease models [13]. Meanwhile, the administration of butyrate (or sodium butyrate) has been shown to suppress neuroinflammation and mitochondrial impairment in obesity models [14] and improve cognitive decline via the alleviation of hippocampal mitochondrial impairment in diabetes models [15]. Regarding HMPA, in addition to suggestions of amyloid-β aggregation inhibition [16], a randomized controlled trial combining food containing fermented rice bran with light exercise reported improved cognitive function in the elderly [17]. Furthermore, salmon milt-derived DNA (nucleic acid component) has been reported to improve memory task performance [18] and provide neuroprotective effects [19]. Additionally, *C. butyricum* has been reported to improve the gut microbiota [20,21]. In a previous study by our research group using food containing *C. butyricum* and HMPA, although improvement in bowel habits was not clear in the overall population [22], significant improvement was reported in an analysis targeting those with constipation tendencies [23]. Based on these findings, we set cognitive function as the primary outcome in this study and measured bowel habit indices to evaluate the possibility of gut environment improvement. In addition, probiotic intervention containing butyrate-producing bacteria has been reported to improve postprandial glycemic control in patients with type 2 diabetes [24]. Furthermore, intervention with *C. butyricum* has been shown to improve metabolic abnormalities and reduce insulin resistance via the gut microbiota in obesity models [25–27], and hepatoprotective effects via the regulation of short-chain fatty acid homeostasis have also been reported [28]. Regarding HMPA, human intervention trials have reported improvements in glucose metabolism [29], lipid profiles (LDL cholesterol) [30], and reduction of visceral fat area [31], with suggested mechanisms including the improvement of hepatic lipid metabolism via GPR41 [32]. A placebo-controlled double-blind study aimed at improving liver function indices has also been reported for salmon milt-derived DNA [33]. Based on the above, this study also exploratorily

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evaluated metabolic-related markers to examine the multifaceted efficacy and part of the mechanism of action of the Test food.

2. Materials and Methods

2.1. Study Design and Ethical Considerations

This study was a randomized, double-blind, placebo-controlled, parallel-group comparison trial conducted to examine the effects of Test food intake on cognitive function and bowel habits. The overall flow of the study, visit timing, and timing of each assessment are shown in Figure 1. This trial was conducted in accordance with the Declaration of Helsinki (2024 revision) and the ethical principles of the "Ethical Guidelines for Medical and Biological Research Involving Human Subjects" (Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labour and Welfare; Ministry of Economy, Trade and Industry). The study was approved by the Ethics Review Committee of Chiyoda Paramedical Care Clinic (IRB No.: 15000088, Approval Date: 21 March 2025) and registered with the UMIN Clinical Trials Registry (UMIN000057405) prior to study initiation. Recruitment and management of study participants were conducted by CPCC Co., Ltd., and interventions and evaluations were performed at Chiyoda Paramedical Care Clinic. The study period was from 31 March 2025 to 5 December 2025, during which the study was conducted with due consideration for the safety and protection of the human rights of the participants.

The required sample size for this study was calculated based on the results of a 12-week two-dose pilot study with a pre-post design (n = 10) [34] using food containing the same ingredients, focusing on the standardized score of Cognitrax (Health Solution, Inc., Shibuya-ku, Tokyo, Japan) Composite Memory. Assuming a significance level of 0.05 and a power of 0.8 in an analysis considering pre-intervention as the control and post-intervention as the test group, the required sample size was estimated to be 58 (29 per group). Considering the relatively older age of the subjects and the long duration of the study (12 weeks), we anticipated some dropouts and set the final target enrollment at 80 (40 per group).

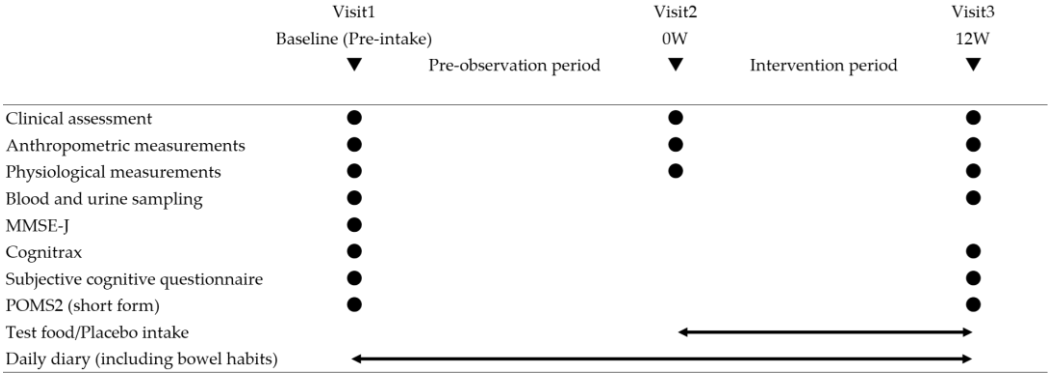

Figure 1. Study schedule. This figure illustrates the overall study schedule, including the baseline assessment (pre-intake; Visit 1), the 2-week pre-observation period, and the 12-week intervention period. Week 0 (Visit 2) indicates the start of Test food/Placebo intake, and week 12 corresponds to Visit 3. The timing of each assessment-clinical assessment (including medical interviews to identify adverse events), anthropometric measurements, physiological measurements, laboratory tests (blood and urine, including serum BDNF), MMSE-J, Cognitrax, the subjective cognitive questionnaire, and POMS2 (short form)-is indicated by filled circles. Continuous activities are indicated by black arrows: Test food/Placebo intake from week 0 through week 12 and daily diary recording from the start of the 2-week pre-observation period through the week 12 assessment.

2.2. Participants

In this study, participants who met the inclusion criteria and did not meet any of the exclusion criteria were enrolled.

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Inclusion criteria were: (1) men and women aged 55–79 years at the time of informed consent; (2) awareness of forgetfulness or having been told that they are forgetful; (3) a Mini-Mental State Examination Japanese version (MMSE-J) score ≥ 24 at baseline (pre-intake; Visit 1); and (4) ability to understand the study procedures and provide written informed consent.

Exclusion criteria were: (1) habitual use (≥3 times/week) of Foods for Specified Health Uses, Foods with Function Claims, or health foods (including supplements) that may affect the study outcomes within 3 months before consent; (2) inability to discontinue such products from the time of consent; (3) use of medications that may affect the study outcomes (e.g., dementia medications, antibiotics, laxatives, intestinal regulators) that could not be restricted during the study period; (4) diagnosis of dementia and receiving treatment; (5) extremely irregular dietary habits or lifestyle rhythms; (6) participation in another clinical trial (pharmaceuticals or health foods), within 4 weeks after completing a trial, or planned participation after consenting to this study; (7) heavy alcohol consumption; (8) self-reported color blindness (including prior diagnosis); (9) history or current serious disease of the brain, heart, liver, kidney, gastrointestinal system, etc.; (10) allergy to medicines or foods (especially salmon); (11) Those who have donated component blood or 200 mL whole blood within 1 month prior to the start of the study; (12) Men who have donated component blood or 400 mL whole blood within 3 months prior to the start of the study; (13) Women who have donated component blood or 400 mL whole blood within 4 months prior to the start of the study; (14) Men whose total blood sampling volume, including the planned amount for this study, would exceed 1200 mL in the 12 months prior to the start of the study; (15) Women whose total blood sampling volume would exceed 800 mL in the 12 months prior to the start of the study; and (16) Those judged inappropriate for participation in this study by the principal investigator or sub-investigator.

2.3. Randomization and Blinding

Among those who consented to participate, met the inclusion criteria, and did not meet the exclusion criteria, those with no clinical abnormalities at baseline (pre-intake; Visit 1) and judged by the principal investigator to have no issues participating were enrolled. After enrollment, an allocation manager from an independent third-party organization not directly involved in the study assigned participants to either the Test food or Placebo group using a computer-generated randomization sequence, with allocation factors including age, sex, Cognitrax (Composite Memory standardized score), and daily defecation frequency calculated from the 2-week pre-observation period prior to week 0. An allocation list was then created.

The Test food and Placebo capsules were identical in appearance, color, and packaging, and were matched in flavor and odor to ensure they were indistinguishable. The study sponsor (Nicorio Co., Ltd.), which prepared and supplied the study products, created a Study Product Identification Table linking study product codes to product type (Test food or Placebo), which was sealed and stored. Simultaneously, the study products labeled only with the codes were sent to the study product manager at the contract research organization. The allocation manager provided the allocation list to the study product manager. The study product manager at the contract research organization was a person not involved in the study conduct, and the allocation list was strictly managed and was inaccessible to investigators and other study personnel until key opening. At the time of key opening, the study sponsor disclosed the Study Product Identification Table, and the study product manager disclosed the allocation list. Through these procedures, blinding was appropriately maintained until key opening.

2.4. Intervention

The Test food was a processed capsule food containing *C. butyricum* (1.4×10^7 CFU), fermented rice bran-derived HMPA (Maruzen Pharmaceuticals Co., Ltd., 11.5 mg), and salmon milt-derived DNA (Maruha Nichiro Corporation, 45 mg). Participants were instructed to ingest one capsule daily with water or warm water at any time for 12 weeks. The Placebo capsule contained no active

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ingredients and was matched to the Test food in appearance and flavor. Details of the nutritional components and composition of the Test food and Placebo are shown in Supplementary Table S1.

2.5. Outcome Measures

2.5.1. Cognitive Function (Primary Outcome)

Cognitive function, the primary outcome, was assessed using Cognitrax [35]. Cognitrax is a cognitive function test battery developed based on the cognitive testing technology of CNS Vital Signs, capable of comprehensively evaluating multiple neurocognitive domains. In this study, tests were conducted following standardized procedures including Verbal Memory (VBM), Visual Memory (VIM), Finger Tapping (FTT), Symbol Digit Coding (SDC), Stroop Test (ST), Shifting Attention (SAT), and Continuous Performance Test (CPT). Standardized scores for Neurocognitive Index (NCI), Composite Memory, VBM, VIM, Psychomotor Speed, Reaction Time, Complex Attention, Cognitive Flexibility, Processing Speed, Executive Function, Simple Attention, and Motor Speed were used for analysis. In addition, standardized scores and changes from baseline were calculated for each subtest such as VBM, VIM, FTT, SDC, ST, SAT, and CPT. Cognitrax assessment was performed at baseline (pre-intake; Visit 1) and at week 12 (Visit 3).

2.5.2. Subjective Cognitive Function

Subjective cognitive function was evaluated using a self-administered dementia checklist. This questionnaire is a self-administered evaluation scale designed to capture subjective changes in memory, attention, and activities of daily living, developed and validated in a survey of community-dwelling elderly people in Japan [36]. Participants answered this checklist at baseline (pre-intake; Visit 1) and at week 12 (Visit 3). The obtained responses were scored based on a predetermined scoring method, and the total score was used as an indicator of change in subjective cognitive function for analysis.

2.5.3. Psychological State

Psychological state was evaluated using the Profile of Mood States 2 (POMS2) Japanese Short Form [37–39], a standard psychological test evaluating acute and transient mood states and emotions, widely used as an indicator of mood change in clinical and intervention studies. The POMS2 Short Form consists of 35 items rated on a 5-point Likert scale (0–4) and evaluates five negative mood scales (Anger-Hostility, Confusion-Bewilderment, Depression-Dejection, Fatigue-Inertia, Tension-Anxiety) and two positive mood scales (Vigor-Activity, Friendliness). The Total Mood Disturbance (TMD) score, a measure of overall mood state, was calculated by subtracting the Vigor-Activity score from the sum of the five negative mood scores.

2.5.4. Biochemical Parameters

Blood pressure, pulse rate, and body weight were measured at baseline (pre-intake; Visit 1), at week 0 (Visit 2), and at week 12 (Visit 3). Hematological and biochemical tests were performed using blood samples collected at baseline (pre-intake; Visit 1) and at week 12 (Visit 3); participants fasted for 4 h prior to sampling (water allowed). Hematological items included white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), and platelet count (Plt). Biochemical items included total protein (TP), albumin (Alb), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LD), alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT), creatine kinase (CK), urea nitrogen (BUN), creatinine (CRE), uric acid (UA), electrolytes (sodium, chloride, potassium, calcium), lipid-related items (total cholesterol (T-Cho), Low Density Lipoprotein-Cholesterol (LDL-C), High Density Lipoprotein-Cholesterol (HDL-C), triglycerides (TG)), glucose (GLU), and HbA1c. Urinalysis evaluated protein, glucose, bilirubin, occult blood, and urobilinogen. Hematological, biochemical,

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and urinalysis tests were conducted by BML, Inc. Serum BDNF was measured by ELISA at LSI Medience Corporation using serum samples collected at baseline (pre-intake; Visit 1) and at week 12 (Visit 3) after serum separation.

2.5.5. Bowel Habits

Bowel habits were evaluated using a daily life diary kept by participants. Evaluation items included defecation frequency, number of defecation days, stool amount, stool characteristics (shape and hardness), sensation of incomplete evacuation, abdominal pain, and odor. Participants were instructed to record their bowel habits daily in the diary during the 2-week pre-observation period and the 12-week intervention period. Based on the recorded information, weekly defecation indices were calculated as weekly mean values and used for between-group comparisons and analysis of changes over time.

2.6. Compliance and Safety

Treatment adherence was evaluated based on the daily life diaries recorded by participants and the number of unconsumed capsules collected at visits. Participants were instructed to record study product intake status and physical condition in the diary. The treatment adherence rate was calculated as the percentage of actual intake days to the prescribed intake days. Adverse events (AEs) occurring during the study period were recorded based on participant reports and physician examination results. The principal investigator evaluated the severity of AEs and their causal relationship with the study product.

2.7. Statistical Analysis

Statistical analyses were conducted according to a prespecified statistical analysis plan. The Per-Protocol Set (PPS) was used for primary and secondary outcomes, and the Full Analysis Set (FAS) was used for safety evaluation. Missing values were not imputed. For serum BDNF, when results were outside the assay's quantification range (below the LLOQ or above the ULOQ), the reference values reported by the laboratory were used for analysis. The primary outcome was analyzed using an unpaired t-test (Student's t-test for equal variances and Welch's t-test for unequal variances). For other continuous variables, normality was assessed using the Shapiro–Wilk test. If normality was confirmed, Student's t-test or Welch's t-test was used as appropriate; if not, the Wilcoxon rank-sum test was used for between-group comparisons. Paired t-tests or Wilcoxon signed-rank tests were used for within-group comparisons, as appropriate. Ordinal and categorical variables were analyzed without normality testing using nonparametric methods (e.g., Wilcoxon rank-sum test for ordinal data and Fisher's exact test for categorical data). Bonferroni correction was applied to the within-group comparisons of defecation indices to account for multiple comparisons. To further explore the effect of the Test food on the primary outcome, a subgroup analysis was conducted after database lock. A subgroup analysis was performed based on age at baseline (pre-intake; Visit 1) ≥ 65 years and an MMSE-J total score of 24–27. All statistical tests were two-sided, and the significance level was set at $p < 0.05$. IBM SPSS Statistics, Version 30.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Figures were generated using R (Version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) with ggplot2 (Version 3.5.1; tidyverse version 2.0.0) and cowplot (Version 1.1.3). Changes from baseline (Δ) were visualized as violin plots.

2.8. Use of Generative AI

ChatGPT (OpenAI) was used solely to assist with English phrasing and improve the clarity of the manuscript. It was not used to generate scientific content, study design, data, or figures, nor to perform data analysis or interpret the results. All output was reviewed, edited, and verified by the authors, who take full responsibility for the final content.

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3. Results

3.1. Participant Flow and Analysis Sets

The participant flow and breakdown of analysis sets in this study are shown in Figure 2. A baseline (pre-intake; Visit 1) assessment was conducted on 178 individuals who provided written consent. Based on the selection/exclusion criteria, baseline assessment results, cognitive function tests, and bowel habits, 80 individuals were selected as the final subjects for inclusion. The 80 included subjects were allocated to the Test food group and the Placebo group (40 per group) using age, sex, Cognitrax Composite Memory standardized score, and defecation frequency during the pre-observation period as allocation factors. After allocation, all 80 subjects started ingesting the assigned study products (Test food or Placebo). Three subjects discontinued during the study period, and 77 subjects completed the study (Placebo group: 38, Test food group: 39). No subjects met the FAS exclusion criteria, so the Intention to Treat (ITT) and FAS analysis sets consisted of 80 subjects (40 per group). However, in the PPS analysis, 6 subjects meeting exclusion criteria were excluded, resulting in 74 subjects (37 per group) for analysis. Details of discontinued cases and excluded cases are shown in Supplementary Table S2 and Supplementary Table S3.

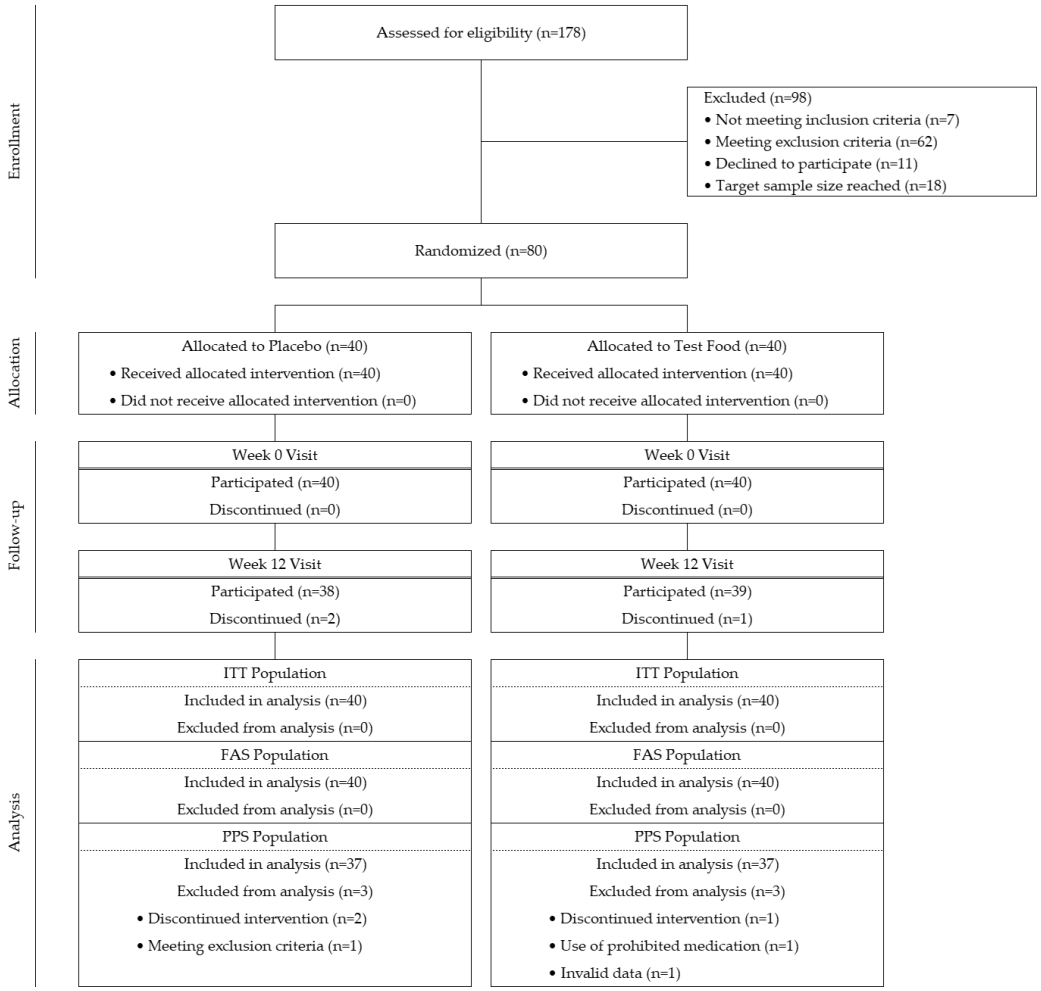

Figure 2. CONSORT flow diagram.

3.2. Baseline Characteristics

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Baseline characteristics of the participants are shown in Table 1. No significant differences were observed between the Test food and Placebo groups in age, sex, anthropometric measurements, cognitive function indices, bowel habits, or metabolic markers including glucose metabolism, liver function, and lipid-related indices. Statistical methods used for baseline comparison are indicated in the footnotes of Table 1.

Table 1. Baseline characteristics of the participants.

Items (Unit)	Placebo (n = 37) Mean (SD)	Test food (n = 37) Mean (SD)	*p*-value
Age (years)	65.6 (4.3)	65.6 (4.1)	0.9265 [a]
Male	24	23	1.0000 [b]
Female	13	14	
Height (cm)	164.74 (8.97)	164.66 (7.94)	0.9684 [c]
Body weight (kg)	63.23 (11.61)	62.10 (9.18)	0.6431 [c]
BMI (kg/m^2)	23.15 (2.89)	22.85 (2.59)	0.8161 [a]

Table 1. *Cont.*

Variable	Placebo (n = 37) Mean (SD)	Test food (n = 37) Mean (SD)	*p*-value
MMSE-J score	27.4 (1.9)	27.8 (1.5)	0.3688 [a]
NCI (Cognitrax)	102.4 (8.2)	103.1 (9.1)	0.7480 [c]
Composite Memory (Cognitrax)	92.9 (16.5)	96.1 (19.9)	0.4481 [c]
BDNF (pg/mL)	67.05 (50.37)	330.85 (968.43)	0.1520 [a]
Defecation frequency (times/week)	7.9 (3.9)	7.9 (2.8)	0.7905 [a]
Glucose (mg/dL)	96.0 (8.2)	96.1 (7.4)	0.9645 [c]
HbA1c (NGSP, %)	5.50 (0.28)	5.57 (0.32)	0.2633 [a]
Aspartate aminotransferase (U/L)	23.1 (4.6)	22.5 (5.9)	0.6456 [c]
Alanine aminotransferase (U/L)	18.5 (6.9)	19.6 (8.3)	0.5190 [c]
Gamma-glutamyl transferase (U/L)	26.2 (12.4)	24.2 (13.6)	0.3520 [a]
Total cholesterol (mg/dL)	210.7 (26.2)	206.7 (25.9)	0.5110 [c]
Triglycerides (mg/dL)	105.9 (57.1)	93.5 (37.5)	0.5377 [a]
HDL cholesterol (mg/dL)	66.2 (18.6)	66.7 (15.3)	0.7661 [a]
LDL cholesterol (mg/dL)	123.6 (24.4)	122.9 (27.4)	0.9146 [c]

Values are presented as mean (SD) or n. P values were calculated using the following tests: [a] Wilcoxon rank-sum test; [b] Fisher's exact test; [c] Student's t-test.

3.3. Effects on Cognitive Function (Primary Outcome)

Cognitive function, the primary outcome, was evaluated using the PPS analysis set. In the overall analysis, no significant difference was observed between the Test food and Placebo groups in the standardized score of Cognitrax Composite Memory. On the other hand, the standardized score for Processing Speed showed an improving trend in the Test food group, though it did not reach statistical significance. Among these cognitive function indices, the standardized score for correct responses in the SDC subtest significantly improved in the Test food group compared with the Placebo group. The changes (Δ) in these major cognitive function indices are shown in Figure 3. Results for other Cognitrax indices and subtests are shown in Supplementary Table S4.

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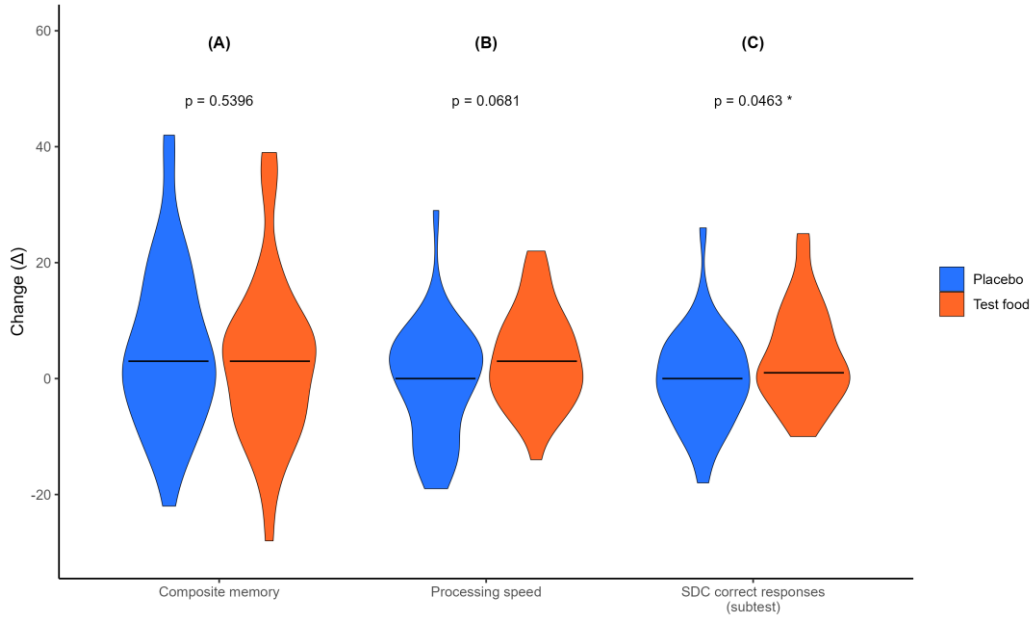

Figure 3. Changes in selected cognitive function scores assessed by Cognitrax. Changes from baseline (Δ) in (A) Composite memory, (B) Processing speed, and (C) SDC correct responses (subtest) after 12 weeks of intervention are shown for the Test food and Placebo groups. Δ indicates the change from baseline (week 12 minus baseline). Values represent mean ± SD. Between-group comparisons were performed using changes from baseline. *p < 0.05.

3.4. Subgroup Analysis of Cognitive Function

To further examine the effects of the Test food on the primary outcome, a subgroup analysis was conducted. The subjects for this analysis were those aged ≥65 years at baseline (pre-intake; Visit 1) with an MMSE-J total score of 24–27. Seventeen subjects meeting these conditions (Placebo: n=10, Test food: n=7) were extracted and subjected to subgroup analysis. In this population, no significant between-group differences were observed in major cognitive indices at baseline (pre-intake; Visit 1). Results of the analysis in this stratum showed that the standardized score for VBM in Cognitrax significantly increased in the Test food group compared with the Placebo group. Additionally, the standardized score for correct hits in the VBM subtest also significantly increased in the Test food group. Conversely, different trends were observed for indices related to information processing efficiency. The standardized score for Reaction Time showed a decreasing trend in the Test food group, and the standardized score for errors in the SDC subtest significantly decreased in the Test food group. These results suggest that the effect of Test food intake on cognitive function may not be uniform across cognitive domains. The changes (Δ) in major cognitive function indices observed in this subgroup analysis are shown in Figure 4. Results for other Cognitrax indices and subtests are shown in Supplementary Table S5.

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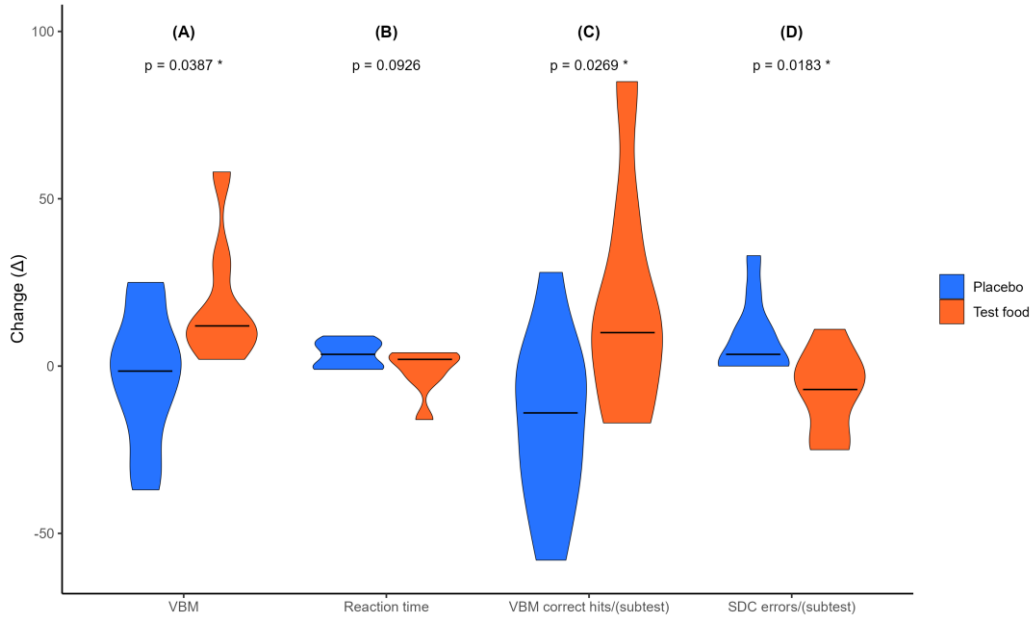

Figure 4. Changes in selected cognitive function scores in the subgroup. Changes from baseline (Δ) in (A) VBM, (B) Reaction time, (C) VBM correct hits (subtest), and (D) SDC errors (subtest) after 12 weeks of intervention are shown for participants aged ≥65 years with baseline MMSE-J scores of 24–27. Δ indicates the change from baseline (week 12 minus baseline). Values represent mean ± SD. Between-group comparisons were performed using changes from baseline. *$p < 0.05$.

3.5. Effects on Metabolic and Biochemical Parameters

Metabolic and blood biochemical indices were evaluated using the PPS analysis set. In the overall analysis, among glucose metabolism indices, the change (Δ) in fasting plasma GLU showed a significant decrease in the Test food group compared with the Placebo group, whereas the change (Δ) in HbA1c (NGSP) showed a significant attenuation of the increase in the Test food group compared with the Placebo group. Regarding liver function indices, the change (Δ) in γ-GT showed a significant decrease in the Test food group compared with the Placebo group. Furthermore, the change (Δ) in UA showed a significant attenuation of the increase in the Test food group compared with the Placebo group. The changes (Δ) in major items showing between-group differences among these metabolic and blood biochemical indices are shown in Figure 5. Results for other blood biochemical test items are shown in Supplementary Table S6.

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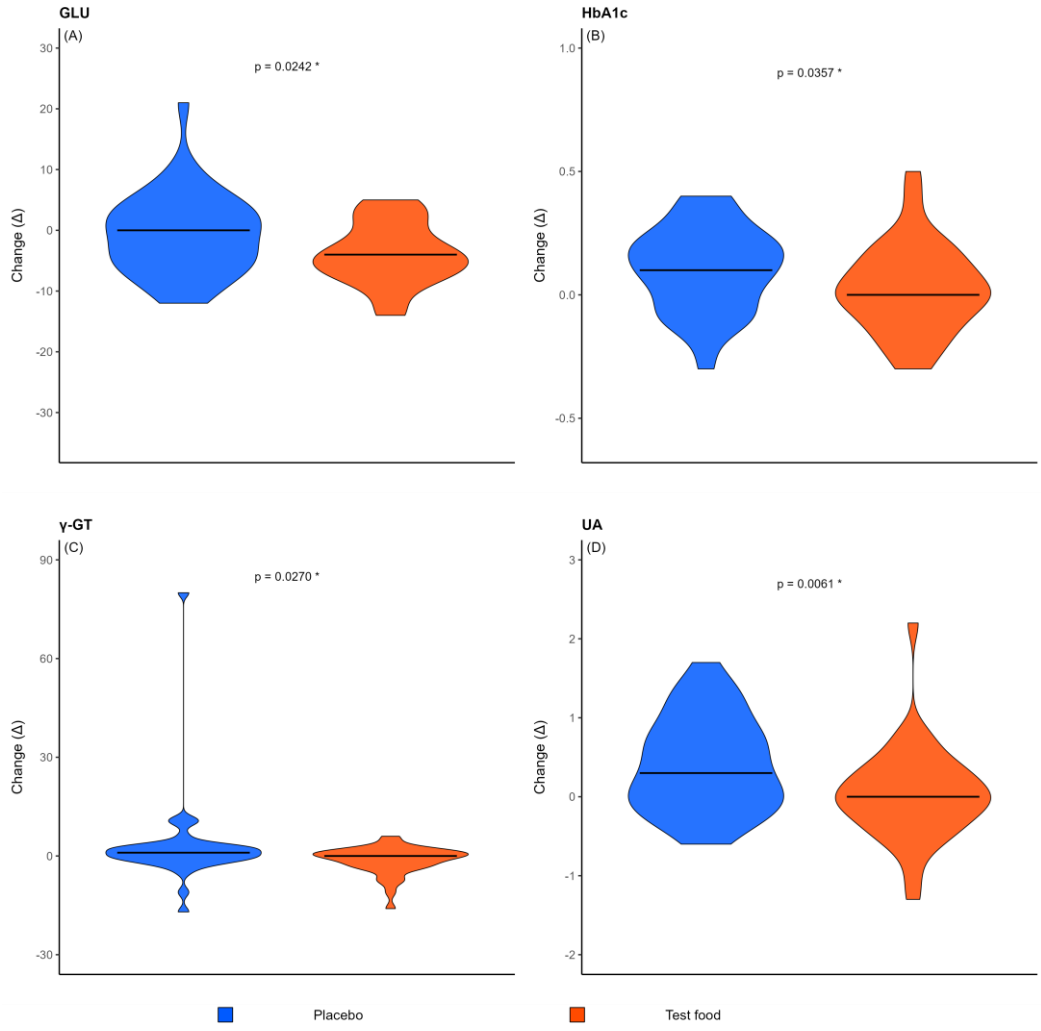

Figure 5. Changes in selected metabolic and biochemical parameters. Changes from baseline (Δ) in (A) fasting plasma GLU, (B) HbA1c (NGSP), (C) γ-GT, and (D) UA after 12 weeks of intervention are shown for the Test food and Placebo groups. Δ indicates the change from baseline (week 12 minus baseline). Values represent mean ± SD. Between-group comparisons were performed using changes from baseline. *$p < 0.05$.

3.6. Other Secondary Outcomes

3.6.1. Subjective Cognitive Function

Analysis of the self-administered cognitive function evaluation results showed no significant differences between the Test food and Placebo groups in the change of each evaluation item. However, in actual values after 12 weeks, the proportion of participants answering, "Can do without problems" to the item "Can you handle deposits/withdrawals and pay rent/utility bills by yourself?" was significantly higher in the Test food group compared to the Placebo group.

3.6.2. Psychological State

Results of psychological state evaluation using the POMS2 Japanese Short Form showed no significant differences between the Test food and Placebo groups in any of the scales: Tension–Anxiety, Depression–Dejection, Anger–Hostility, Vigor–Activity, Fatigue–Inertia, Confusion–Bewilderment, or Friendliness.

3.6.3. Bowel Habits

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Analysis of bowel habit indices including defecation frequency, number of defecation days, stool characteristics, sensation of incomplete evacuation, abdominal pain, and odor revealed no consistent significant differences between the Test food and Placebo groups in any index.

3.7. Compliance and Safety

Treatment adherence during the study period was high, with no difference observed between groups. No serious adverse events (SAEs) were reported during the study, although some participants discontinued the study. The number of subjects experiencing adverse events in the safety evaluation (FAS) was 12/40 (30%) in the Placebo group and 10/40 (25%) in the Test food group, with no significant difference between groups (Fisher's exact test, $p = 0.8027$). Side effects (events for which a causal relationship with the study product could not be ruled out) were not observed in either group. Furthermore, in safety evaluations including hematological and blood biochemical tests, no clinically problematic changes attributable to study product intake were observed (Supplementary Table S6). In addition, blood pressure, pulse rate, and body weight were assessed at week 0 (Visit 2) for safety monitoring; no clinically relevant changes or between-group differences were observed (data not shown).

4. Discussion

This study evaluated the effects of consuming a functional food containing *C. butyricum*, HMPA, and salmon milt-derived DNA on cognitive function and bowel habits in middle-aged and older adults. In the overall population, indices related to attention and processing speed (SDC correct responses) significantly improved, and processing speed scores also showed an improving trend. Furthermore, in the high-risk population (aged ≥65 years and MMSE-J 24–27), significant improvements in verbal memory scores and related indices were observed. Maintaining and improving verbal memory in the elderly is important for suppressing the progression from MCI to dementia and may contribute to maintaining patients' QOL and extending independent living. Indeed, reports indicate that QOL decline begins at the MCI stage and worsens significantly upon progression to dementia [40]. Indeed, estimates suggest that delaying the onset of dementia by just one year could reduce the global number of dementia patients by more than 6 million by 2050 [3], suggesting that the social and economic impact of such interventions would be substantial. On the other hand, no significant changes were observed in bowel habit indices. This may be partly due to the lack of constipation tendencies in the study population. Additionally, exploratory blood biochemical tests showed improvements in metabolic markers such as fasting plasma glucose, HbA1c, γ-GT, and uric acid, suggesting that changes in metabolic function may contribute to the improvement of cognitive function.

Cognitrax, the cognitive function test used in this study, is a Japanese version of the US-developed computerized cognitive test battery "CNS Vital Signs." It has a configuration similar to conventional neuropsychological tests, and high reliability and validity have been reported. For example, test-retest reliability has been confirmed with high intraclass correlation coefficients of 0.7 or higher for many indices in both healthy individuals and MCI patients [41]. Validation studies by the developers of CNS Vital Signs, the basis of Cognitrax, also confirmed that test-retest reliability is comparable to equivalent conventional tests and that concurrent validity of each test is established [35]. Thus, Cognitrax exhibits characteristics very similar to conventional paper-and-pencil tests and is positioned as a useful objective cognitive evaluation tool. In this study, significant improvement was observed in the SDC correct response score, an indicator of attention and information processing speed, in the overall population, and the standardized score for Processing Speed also showed an improving trend. Conversely, no clear difference was confirmed in memory domains such as Composite Memory. The MMSE-J used for screening in this study has a maximum score of 30, with a score of 23 or lower generally considered strongly suspicious of dementia [42]. Meanwhile, scores of 24–27 are reported to indicate suspicion of MCI [42]. According to the World Alzheimer Report 2015, the incidence of dementia after age 65 rises more than 1.5 times compared to ages 60–64 and

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tends to increase steadily with age [43]. Based on this, we conducted a subgroup analysis in a high-risk group aged 65 and over with MMSE-J scores of 24–27. The results showed that the standardized score for VBM and the number of correct hits significantly improved in the Test food group, indicating that intervention effects may appear more clearly in populations at high risk of cognitive decline.

Several previous findings related to cognitive function have been reported for each component of the complex functional food used in this study. The butyrate-producing bacterium *C. butyricum* has been shown to contribute to the suppression of inflammatory responses and neuroprotection by regulating the gut-brain axis through modification of the gut microbiota and SCFA production, with reported improvements in cognitive function indices in obesity and Alzheimer's disease models [12–15]. Regarding HMPA, a component derived from fermented rice bran, involvement in neurodegeneration-related mechanisms such as inhibition of amyloid-β aggregation has been suggested [16], and a randomized, double-blind, placebo-controlled trial combining food containing fermented rice bran with light exercise reported improved cognitive function in the elderly [17]. Furthermore, salmon milt-derived DNA (nucleic acid component) has been reported to have effects related to memory and learning, such as improved memory task performance accompanied by increased nucleoside levels in the hippocampus and neuroprotective effects under oxidative stress conditions [18,19]. These findings suggest the possibility that these components support cognitive function from multiple action points, including indirect pathways via changes in the gut environment and systemic state, in addition to direct central effects of single components.

Consistent with these previous findings, the exploratory subgroup analysis in this study (age ≥ 65 years and MMSE-J 24–27) showed improvements in VBM indices in the Test food group. In contrast, indices related to processing efficiency, such as reaction time and SDC errors, showed mixed changes and did not demonstrate consistent improvement in the Test food group. Cognitive function is multifaceted and involves multiple brain regions and neural networks. Memory function is known to be related to brain regions centered on the medial temporal lobe, particularly the hippocampus [44,45], and structural and functional characteristics of the left hippocampus strongly influence verbal episodic memory [46,47]. In contrast, information processing speed and reaction time have been linked to frontal lobe and frontal–subcortical circuits, and age- and disease-related changes such as frontal lobe degeneration and white matter lesions have been associated with declines in these functions [48,49]. The domain-specific pattern observed in this study may be consistent with such prior observations; however, because neurophysiological measures (e.g., EEG) or neuroimaging were not collected, any interpretation regarding underlying neural substrates should be considered exploratory. Future studies incorporating objective brain-based measures are warranted to clarify whether the Test food preferentially affects specific cognitive domains and to identify populations most likely to benefit.

In this study, we set bowel indices such as defecation frequency and subjective bowel symptoms as evaluation items as part of exploring the relationship between the gut environment and brain function (gut-brain axis). Regarding *C. butyricum* contained in the Test food, improvement effects on the gut environment have been reported for some strains such as CBM 588 [20,21], and administration of GKB7 has been reported to improve symptoms in constipation model animals [50]. Based on these findings, similar effects were expected in this study. However, no statistically significant difference was observed between the Test food and Placebo groups in major bowel indices such as defecation frequency and stool characteristics. Although transient between-group differences were observed in items such as abdominal pain during defecation and sensation of incomplete evacuation at some evaluation points, no continuous and consistent improvement trend was shown. These changes are considered more likely due to individual differences or temporary fluctuations caused by external factors rather than the effect of the intervention. Furthermore, this study did not include constipation tendency in the inclusion criteria, and it is inferred that many participants had relatively stable bowel conditions before intervention. Unlike this Test food (butyrate bacteria + HMPA + DNA), a previous study by our research group using food containing *C. butyricum* and HMPA also reported no clear

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bowel improvement in the overall population [22], while significant improvement was reported in the population with constipation tendencies [23]. Thus, in a population where bowel conditions are stable before intervention, the downstream effect on cognitive function via improvement of the gut environment, i.e., the influence of the gut-brain axis, may have been difficult to capture with bowel indices. In the future, it is necessary to examine the mechanism mediated by the gut-brain axis in more detail in studies including subjects with bowel abnormalities or dysbiosis.

In contrast to bowel indices, significant improvements were observed in the Test food group in several blood biochemical indices related to metabolic function in this study. In particular, decreases in fasting plasma glucose and γ-GT, and suppression of increases in HbA1c and UA were observed compared to the Placebo group. Although all changes were mild, these results suggest improvements in glucose metabolism and liver function, consistent with the hypothesis of the mechanism of action based on the ingredients of the Test food. Intervention with multiple probiotic formulations containing butyrate-producing bacteria has been reported to improve glucose metabolism indices such as blood glucose after glucose tolerance tests and HbA1c [24], and animal studies have also shown improvement effects on obesity, diabetes, and fatty liver [25–28]. As a mechanism of action, it has been shown that butyrate produced by butyrate bacteria may regulate energy metabolism in the liver by activating bile acid receptors such as TGR5, contributing to the improvement of glucose metabolism abnormalities derived from fatty liver [28]. Furthermore, protective effects against cognitive impairment caused by obesity have also been reported [12]. Regarding HMPA, effects of improving glucose and lipid metabolism and reducing body fat have been confirmed in human intervention trials [29–31], and it is shown that these effects may be due to mechanisms mediated by GPR41, a short-chain fatty acid receptor [32]. Also, salmon milt-derived DNA has been reported to have hepatoprotective effects [33]. In this study, although improvement in cognitive function was limited to some domains, particularly verbal memory in the high-risk group, the parallel improvement in metabolic markers suggests that improvement in metabolic function may play a certain role in the maintenance and enhancement of cognitive function. Recent studies have reported that metabolic abnormalities such as insulin resistance are involved in age-related cognitive decline and the risk of developing dementia. For example, it has been shown that higher insulin resistance is associated with significantly lower performance in verbal fluency tasks [51], smaller gray matter volume in brain regions vulnerable in Alzheimer's disease [52], and reduced volume in hippocampal subregions [53]. Furthermore, a cohort study targeting patients with type 2 diabetes reported that the risk of developing dementia was reduced to one-fifth in the group treated with metformin compared to the non-treatment group [54]. The improvement in metabolic indices observed in this study, especially changes in glucose-related indices, is consistent with these previous findings, suggesting that the Test food may support part of cognitive function through metabolic improvement in middle-aged and older adults.

The improvements in metabolic indices and cognitive function observed in this study are likely not the effect of a single component but the result of the complementary contribution of multiple formulated components via their respective mechanisms of action. *C. butyricum* contributes to metabolic improvement via short-chain fatty acids along with the regulation of the gut environment, and HMPA may improve insulin sensitivity through glycolipid metabolism. Furthermore, salmon milt-derived DNA may have been involved in the improvement of liver function indices, and it is thought that these actions affected the metabolic balance in a multilayered manner. It is also conceivable that such metabolic improvement led to the support of brain function including memory-related regions such as the hippocampus. In the future, it is necessary to clarify the degree of contribution of each component by verifying them individually or in different combinations.

Limitations of this study include the limited number of cases, lack of adjustment for multiple comparisons other than for bowel indices, and the short cognitive function evaluation period of 12 weeks. Also, the association between metabolic indices and cognitive function does not indicate a causal relationship. In the future, long-term intervention trials targeting larger populations and analyses including detailed metabolic and inflammation markers are necessary. Furthermore,

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verification of gut environment improvement and cognitive function changes in populations with constipation tendencies is required.

5. Conclusions

Twelve-week intake of this complex functional food improved indices of attention and processing speed in the overall population, improved verbal memory indices in the high-risk subgroup, and positively affected metabolic markers. However, no clear effects on bowel habits were confirmed. Larger-scale and longer-term verification is needed in the future.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Composition and nutritional content of the Test food and Placebo capsules; Table S2: Reasons for study discontinuation; Table S3: Exclusions from analysis sets (PPS) and reasons; Table S4: Effects on cognitive function outcomes; Table S5: Effects on cognitive function outcomes in the high-risk subgroup; Table S6: Effects on blood biochemistry parameters.

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Institutional Review Board Statement: The study was conducted in accordance with the principles of the Declaration of Helsinki and the Ethical Guidelines for Life Sciences and Medical Research Involving Human Subjects (Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labour and Welfare; and Ministry of Economy, Trade and Industry, Japan), and was approved by the Institutional Review Board of Chiyoda Paramedical Care Clinic (IRB No. 15000088; approval date 21 March 2025). The study was registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (No. UMIN000057405).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to privacy and ethical restrictions.

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Conflicts of Interest: Y.T. and M.I. are employees of NICORIO Co., Ltd. A.U. is an employee of Ortho Corporation. This study was conducted as a collaborative project between NICORIO Co., Ltd. and Ortho Corporation, and both companies provided financial support for the study and were involved in the study design, data analysis and interpretation, and manuscript preparation. S.E. is a medical doctor at Chiyoda Paramedical Care Clinic and was contracted to conduct the clinical trial. Recruitment and management of study participants were conducted by CPCC Co., Ltd., and interventions and evaluations were performed at Chiyoda Paramedical Care Clinic. The decision to publish the results was made by the authors.

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